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Title: Interleukin-1α enhances mast cell growth by a fibroblast-dependent mechanism

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## Abstract

Mast cell hyperplasia is observed in various inflammatory skin diseases. Although the pathogenesis of these conditions remains largely uninvestigated, it is speculated that lesional mediators provide favorable microenvironment for mast cell growth. We have investigated the effect of an inflammatory cytokine, IL-1α, on mast cell growth in a mast cell/fibroblast co-culture system. When mouse bone marrow-derived cultured mast cells (BMMC) were cultured on a NIH/3T3 fibroblast monolayer, IL-1α stimulated mast cell proliferation. However, IL-1α did not stimulate $^{3}$H-thymidine incorporation in BMMC in the absence of fibroblasts. Separation of BMMC from fibroblasts by a permeable micropore membrane reduced the effect of IL-1α. When BMMC were prepared from *W/W$^{v}$*-mice, which lack functional c-*kit*, or when NIH/3T3 fibroblasts were substituted with *Sl/Sl$^{d}$*-derived fibroblasts, which lack membrane-bound stem cell factor (SCF), a lower, but significant, effect of IL-1α was observed. Flow cytometric analysis revealed no enhancement of SCF expression on fibroblasts by IL-1α. Neutralizing antibodies against IL-3, IL-4, IL-10, and nerve growth factor (NGF) showed no inhibition. On the other hand, indomethacin inhibited the effect of IL-1α, and prostaglandin $E_2$ induced mast cell growth in the co-culture. These results indicate that IL-1α stimulates mast cell growth by a fibroblast-dependent mechanism, where SCF/c-*kit* interaction may participate in a major way. The mast cell growth activity induced by this cytokine is, at least in part, attributed to prostaglandins. Inflammatory cytokines may thus contribute to mast cell hyperplasia in skin diseases.

## Introduction

Mast cells release inflammatory chemical mediators, such as histamine, leukotrienes, and prostaglandins, on IgE-mediated stimulation with specific allergen. Mast cells thus play a pivotal role in immediate hypersensitivity reactions. In addition, recent studies have revealed that mast cells produce a wide plethora of cytokines, suggesting their active participation in the late phase reaction and chronic stage of inflammation [1, 2].

Mast cells increase in a variety of inflammatory skin diseases, such as atopic dermatitis, contact dermatitis, and in actinically damaged skin [1]. Repeated topical application of antigens [3, 4], phorbol-myristate-acetate [5], or ultraviolet light irradiation [6] is documented to induce increase in mast cells along with skin inflammation. But the pathogenesis of mast cell accumulation in these conditions remains largely uninvestigated.

Accumulation of mast cells could be attributed to several mechanisms, that is, ingress of mast cell precursors, attraction of surrounding mast cells, prolonged survival, and local proliferation of mast cells. Mitoses of mast cells observed in contact dermatitis [7] and atopic dermatitis [8] suggest that local proliferation is a possible mechanism involved.

Mast cells originate from hematopoietic stem cells in the bone marrow and are distributed to the peripheral connective tissues in premature form. Mast cells proliferate or complete their differentiation in the tissue where they reside, depending on the tissue microenvironment. The mechanism of mast cell growth has been largely investigated in murine system, and recent studies using human system revealed substantial differences between two species. In murine system, IL-3 and stem cell factor (SCF) induce mast cell proliferation and IL-4 enhances the effect of these cytokines. While in human system, SCF is the only known mast cell growth factor to date and IL-3 and IL-4 have little effect.

Thus, the results obtained in studies using murine mast cells could not be simply applied to human system. However, it is common in both species that stromal cells play an important role. Indeed, in mast cell/fibroblast co-culture system, mouse skin-derived fibroblasts maintain and induce differentiation of mast cells of mice [9], as well as mast cells of human [10, 11].

In normal skin, mast cells are usually observed in the upper dermis, around blood vessels and nerves, and mast cells observed in inflammatory skin diseases are frequent in superficial dermis [5, 12, 13]. These suggest that fibroblasts, major cellular component of the dermis, play an important role also *in vivo*, and that this mast cell-fibroblast interaction is affected by local inflammatory mediators.

In this investigation, we studied the effect of an inflammatory cytokine, IL-1α, on mast cell growth in the murine mast cell/fibroblast co-culture system. In skin inflammation, IL-1α is produced by keratinocytes, as well as various infiltrating inflammatory cells, such as macrophages and lymphocytes. Therefore, our results serve to elucidate the pathomechanism of mast cell hyperplasia in inflammatory skin diseases.

## Materials and methods

### Cytokines and antibodies

Recombinant murine IL-1α, IL-3, IL-4, IL-10, and SCF were purchased from R&D systems (Minneapolis, MN). Mouse submaxillary gland-derived 2.5S nerve growth factor (NGF) was purchased from Harbor Bio-Products (Norwood, MA) and Toyobo (Osaka, Japan). Antibodies; goat anti-mouse IL-1α, IL-3, IL-4, were purchased from R&D

systems, anti-mouse IL-10 antibody was purchased from Endogen (Cambridge, MA), goat anti-mouse SCF was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and sheep anti-NGF was purchased from Chemicon International (Temecula, CA). Indomethacin and prostaglandin $E_2$ ($PGE_2$) were purchased from Sigma (St. Louis, MO).

## Cells

Bone marrow-derived cultured mast cells (BMMC) were obtained by culturing bone marrow cells of 6-week-old male mice, WBB6F1-*W/W$^v$* (*W/W$^v$*) and their normal littermates $WBB6F_1$-+/+ (+/+) (Shizuoka Laboratory Animal Center, Shizuoka, Japan), as previously described [14, 15]. Briefly, bone marrow cells suspended at a density of $1x10^6$/ml in αMEM medium (Dainippon Pharmaceutical, Osaka, Japan) supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum, 100 IU/ml penicillin G (Meiji Pharmaceutical Co., Tokyo, Japan) and 100 μg/ml streptomycin (Meiji) (complete αMEM) with 5 ng/ml of IL-3 and 2 ng/ml of IL-4 were cultured at 37°C in a humidified atmosphere of 5% $CO_2$ in air. Non-adherent cells were collected by centrifugation and re-suspended in fresh medium every 7 d. After 4 weeks, more than 95% of the non-adherent cells stained positively with alcian blue.

NIH/3T3 fibroblasts (ATCC No. CRL1658) were maintained in complete αMEM at 37°C in humidified atmosphere of 5% $CO_2$ in air. Mouse skin fibroblasts were prepared from the dermis of +/+-mice. *Sl/Sl$^d$*-3T3 fibroblasts were kindly provided by Dr. Y. Kitamura (Department of Pathology, Osaka University Medical School, Osaka, Japan).

## Co-culture

Co-culture of BMMC with NIH/3T3 fibroblasts was performed as described previously [15] with slight modification. Briefly, $2x10^4$ NIH/3T3 fibroblasts suspended in 0.5 ml of complete αMEM were seeded in each well of four-well multidishes (Nunc, Roskilde, Denmark). Upon reaching confluence, the medium was aspirated and 0.5 ml of medium containing various concentrations of cytokines and $2\text{-}3x10^4$ BMMC were added. The cells were cultured at 37°C in a humidified atmosphere of 5% $CO_2$. The cultures were fed with fresh media every other day.

In a set of experiments, confluent monolayers of fibroblasts were incubated with 50 μg/ml mitomycin C (Kyowa Hakko Kogyo Co., Tokyo, Japan) at 37°C for 30 min. After thorough washing, pretreated monolayers were used for the co-culture.

In the pre-incubation experiment, confluent monolayers of fibroblasts were incubated with IL-1α for 6 h before use in the co-culture. In this experiment, BMMC and fibroblasts were co-cultured for 3 d without medium change.

Mast cell counting in co-culture

Adherent cells in the co-culture wells were treated with PBS containing 0.1% (w/v) trypsin (Difco Laboratories, Detroit, MI) and 0.27 mM EDTA for 10 minutes at 37°C, followed by the addition of one tenth volume of fetal calf serum. The dispersed cells were washed twice with PBS and suspended in 1 ml of PBS. Total number of the cells in the suspension was determined using a hemocytometer. Aliquots of cell suspensions were cytocentrifuged and resulting cell preparations were stained with alcian blue after fixation with Carnoy's solution. The number of mast cells, regarded as alcian blue positive cells,

in co-cultures were calculated using the following formula: number of mast cells/well = A x C/B, where A is total number of the cells in a well counted with hemocytometer, B total number of cells counted on a cytocentrifuge specimen, and C number of alcian blue positive cells in the same specimen.

Determination of histamine content in co-culture

Cultured cells in each co-culture well were denatured by adding $HClO_4$ at a final concentration of 0.25 M, and insoluble proteins were precipitated by centrifugation at 10000 g for 10 min. Histamine concentration in the supernatants was assayed with automated fluorometric-HPLC system (Tosoh Corporation, Tokyo, Japan) as previously described [16].

Separation of mast cells from fibroblasts by using micropore membrane

Mast cells were cultured in the tissue culture inserts where they share the same medium with NIH/3T3 fibroblasts, but separated by a 0.2 μm pore membrane (Anopore membrane™, Nunc). Culture medium was added into both the inner and outer sides of the inserts, and BMMC were seeded into the inner side at the start of the co-culture. Culture medium of outer side was replaced on day 2, 4, and 6, then BMMC in the inserts were harvested on day 7. In this experiment, cell number was determined using a hemocytometer.

$^{3}$H-thymidine incorporation

BMMC were plated in wells ($1x10^4$ cells/well) of 96-well in multiwell plates (Becton Dickinson Labware, Franklin Lakes, NJ) with cytokines or medium alone and incubated for 36 h. Then BMMC were pulsed with 0.5 μCi $^3$H-thymidine (specific activity, 5 Ci/mmol, Dupont-New England Nuclear, Boston, MA) and further incubated for 12 h. The cells were harvested on glass filters and incorporated radioactivity in the cells was assayed in a liquid scintillation counter (LSC5100, Aloka, Tokyo, Japan).

Flow cytometric analysis of SCF expression

Fibroblasts were detached from culture flasks by incubation with PBS containing 5 mM EDTA. Harvested cells were washed and suspended in PBS containing 1 % BSA and 0.1 % sodium azide. After incubation with 10 % normal donkey serum (Chemicon), cells were aliquoted and incubated with goat anti-mouse SCF antibody or normal goat IgG. Cells were stained with FITC-conjugated donkey anti-goat IgG antibody and analyzed in a Becton Dickinson FACScaliber flow cytometer.

Statistics

For statistical analyses, t-test and Bonferroni test were used to compare two groups and more than three groups, respectively.

## Results

Enhancement of mast cell growth by IL-1α in the mast cell/fibroblast co-culture system

When BMMC prepared from +/+-mice were seeded on a confluent monolayer of NIH/3T3 fibroblasts, 44.7 ± 9.3 % (mean ± SD, n=8) of the BMMC adhered to the monolayer within 48 h in the control medium. IL-1α did not significantly alter the proportion of adherent BMMC: 30.2 ± 1. 8 % in the presence of 10 ng/ml of IL-1α. But this cytokine induced proliferation of BMMC after the co-culture of 7 d in a dose-dependent manner (Fig. 1A) and total histamine content paralleled mast cell number in the same experiments (Fig. 1B). When mast cells were cultured in the presence of 10 ng/ml of IL-1α, mast cell number increased in a time-dependent manner during the first 10 d in the co-culture (Fig. 1C) and total histamine content paralleled also in these experiments (data not shown). These indicated that total histamine content could serve as an indicator of mast cell number in the co-culture of 7 d. The mast cell growth enhancing effect of IL-1α was reduced by anti-IL-1α antibody (data not shown).

Direct effect of IL-1α on mast cell proliferation

As IL-1α enhanced mast cell growth in the mast cell/fibroblast co-culture system, we have next examined whether this cytokine stimulates mast cell proliferation directly. When mast cells were cultured without fibroblasts for 48 h, $^{3}$H-thymidine incorporation in the last 12 h was 81 ± 10 cpm, 54 ± 7 cpm, and 7111 ± 598 cpm (mean ± SEM, n=3), in the control medium, 10 ng/ml of IL-1α, and 50 ng/ml of SCF, respectively. The effect of the cytokine was also evaluated by direct cell counting. When $1x10^{5}$ cells/well BMMC were cultured without fibroblast, mast cell number rapidly decreased and viable cells were less than $5x10^{3}$ cells/well after 5 d even in the presence of IL-1α, whereas SCF

induced substantial proliferation of mast cells, $1.6x10^5 \pm 1.4x10^4$ cells/well (mean ± SEM, n=3) were observed after 5 d, in the same experiments. IL-1α does not directly stimulate mast cell proliferation.

Pre-incubation of fibroblasts with IL-1α

As it was suggested that IL-1α induces mast cell growth by a fibroblast-dependent mechanism, we then studied the effect of pretreatment of fibroblasts with IL-1α. Pre-treatment of fibroblasts with 10 ng/ml of IL-1α for 6 h induced a significantly larger mast cell number after 3 d: 27281 ± 669 and 21587 ±1110 cells/well (mean ± SEM, n=3, $p<0.01$), in the co-culture with IL-1α-treated fibroblasts and with control fibroblasts, respectively. This indicates that IL-1α stimulates fibroblasts for the production of mast cell growth factor, and the coexistence of IL-1α was not necessary for the activity of this fibroblast-derived mast cell growth factor. On the other hand, conditioned medium of fibroblasts stimulated with IL-1α for 24 h showed no mast cell growth activity in $^3$H-thymidine incorporation experiments (data not shown).

To further characterize the interaction of mast cells and fibroblasts in the co-culture system, we separated mast cells from fibroblasts by using micropore membrane, which prevented cellular contact but allowed diffusion of soluble mediators. Mast cell growth was observed even when mast cells were separated from fibroblasts, but the effect was significantly lower than that observed without membrane (Fig. 2). For efficient mast cell proliferation in this system, mast cells should locate close to or in contact with fibroblasts.

Effect of mitomycin C treatment of fibroblasts

It is possible that mast cell proliferation is merely the result of fibroblast proliferation, because fibroblasts can maintain mast cells in co-culture [9] and IL-1α stimulates fibroblast proliferation [17]. In dose-response and time course experiments, those shown in Fig. 1, we found that IL-1α increased the mast cell number per fibroblast. This suggests that mast cell growth is not only due to fibroblast proliferation. To confirm this, we treated the fibroblast monolayer with the cytostatic agent, mitomycin C, before their use in the co-culture (Table 1). By the mitomycin C treatment, fibroblast proliferation was blocked, and there was no significant difference in fibroblast numbers after cytokine stimulation. However, mitomycine C treatment did not significantly alter mast cell number after the co-culture, indicating that mast cell growth in this system is not necessarily related to fibroblast proliferation.

Involvement of SCF in IL-1α-induced mast cell growth in the co-culture

Fibroblasts constitutively produce one of the potent mast cell growth factors, SCF, predominantly in membrane-bound form [18]. SCF binds its receptor, c-*kit* product, on mast cells to stimulate proliferation. Littermates of +/+-mice, *W/W*$^{v}$-mice have mutations in the tyrosine kinase domain of c-*kit*, and BMMC derived from *W/W*$^{v}$-mice do not proliferate in response to SCF. To examine whether the SCF/c-*kit* pathway mediated the IL-1α-stimulated mast cell growth, we have used *W/W*$^{v}$-derived BMMC in the co-culture (Fig. 3). Although mast cell number and histamine content after 7 d was lower than that

observed with +/+-BMMC, IL-1α caused significant differences in mast cell number, in parallel with total histamine content, in *W/W*$^{v}$-BMMC/fibroblast co-culture.

The effect of IL-1α was also evaluated in the combination of +/+-derived BMMC and the fibroblasts derived from *Sl/Sl*$^{d}$ mice, which lack membrane bound form of SCF (Fig. 4). Similar to the experiments with *W/W*$^{v}$-derived BMMC, IL-1α caused a significant difference in histamine content in this cell combination, although, the absolute content was much lower than that with NIH/3T3 fibroblasts.

As a partial involvement of SCF was indicated, we then assessed whether IL-1α could enhance SCF expression on fibroblasts by indirect immunofluorescence staining and flow cytometry. Although constitutive expression of SCF on fibroblasts was observed, the expression was not altered by the incubation of fibroblasts with 10 ng/ml of IL-1α for 24 h (data not shown).

We have also examined whether another mast cell growth factor, IL-3, or co-factors for mast cell growth, IL-4 and IL-10 [19, 20], are involved in the cytokine-stimulated mast cell growth. As shown in Table 2, neutralizing antibodies against these factors did not inhibit mast cell growth induced by IL-1α in the co-culture. NGF was reported to induce proliferation of BMMC and development of connective tissue-type mast cells in the co-culture in the presence of IL-3 [21]. However, NGF obtained from several companies failed to induce mast cell growth in the concentration range from 1 pg/ml to 100 ng/ml in our system, in which IL-3 was not supplemented (data not shown).

Involvement of PG in the cytokine-induced mast cell growth in the co-culture

We have used a cyclooxygenase inhibitor, indomethacin, to examine whether this enzyme and prostanoids are involved in the cytokine-stimulated mast cell growth in the co-culture (Fig. 5). Indomethacin significantly reduced the effect of IL-1α on both +/+- and *W/W$^v$*-BMMC. These results indicate that cyclooxygenases are involved in the cytokine-induced mast cell growth in the co-culture, and prostanoids comprise the mast cell growth activity produced by fibroblasts. Indeed, the addition of $PGE_2$ in the co-culture induced significant increase in mast cell number and total cellular histamine (Fig. 6).

Effect of IL-1α on growth of mast cells cultured on skin-derived fibroblasts

We examined whether IL-1α induces proliferation of BMMC cultured on skin-derived fibroblasts as well as NIH/3T3 fibroblasts. Fibroblasts were prepared from the skin of +/+-mice, the strain from which BMMC were prepared, and used in the co-culture. As shown in Fig. 7, IL-1α induced increase in total histamine content, which indicates increase in mast cell number in 7 d culture, in the same concentration range. The cytokine-induced mast cell growth mechanism appears also effective in the co-culture with dermal fibroblasts.

## Discussion

In this study we demonstrated that an inflammatory cytokine, IL-1α induced mast cell growth in BMMC/fibroblast co-culture system (Fig. 1). The effect was observed not only in the co-culture with NIH/3T3 fibroblasts, which is established from mouse embryo, but also in the co-culture with skin-derived fibroblasts prepared from the same strain from

which BMMC were obtained (Fig. 7). As IL-1α is produced by keratinocytes and infiltrating cells in inflammatory lesions, this cytokine-stimulated fibroblast-dependent mast cell growth mechanism may play a role in mast cell hyperplasia observed in chronic skin inflammation.

IL-1α by itself does not stimulate mast cell proliferation directly, but stimulates fibroblasts to produce mast cell growth activity. The considerable reduction in mast cell growth by the separation of BMMC from fibroblasts by micropore membrane (Fig. 2) and no effect of IL-1α-stimulated fibroblast conditioned medium suggest the importance of membrane-associated factor(s) in the mast cell growth. Subsequent experiments using defective cell lines indicated the involvement of SCF, a well characterized mast cell growth factor, which is constitutively produced by fibroblasts predominantly in the membrane-bound form [18]. Employment of *W/W*$^{v}$-derived BMMC (Fig. 3) or *Sl/Sl*$^{d}$-derived fibroblasts (Fig. 4) in the co-culture resulted in lower mast cell number and/or histamine content after 7 d in the presence of IL-1α, suggesting the major role of SCF/c-*kit* pathway in the mast cell growth.

As mast cell growth was observed in the co-culture with mitomycin C-treated fibroblasts (Table 1), mast cell growth is not simply induced by fibroblast proliferation and the consequent increase in SCF. However, flow cytometric analysis revealed that the treatment with IL-1α induced no enhancement of SCF expression on the fibroblasts (data not shown). Although conflicting results were reported about SCF expression on cytokine-stimulated human umbilical vein endothelial cells [22-24], our observation agrees with that of Heinrich et al. [25], who described that SCF expression on human fibroblasts was not altered by IL-1α. Moreover, both cytokines induced a significant

difference in mast cell number and/or histamine content in the co-culture with cells that have a defect in SCF/c-*kit* pathway (Fig. 3, 4). These results indicate the presence of an additional pathway modulated by IL-1α.

Indomethacin inhibited the cytokine-induced mast cell growth in the co-culture (Fig. 5). This suggests that cyclooxygenase, the rate-limiting enzyme of prostanoid synthesis, is involved in the mast cell growth mechanism. Indeed a product of cyclooxygenase pathway, $PGE_2$, induced the growth of mast cells in the co-culture (Fig. 6), indicating a probable role of prostanoids. IL-1α is known to stimulate PG synthesis of fibroblast by inducing the expression of cyclooxygenase-2 [26]. As indomethacin also inhibited the growth of *W/W$^v$*-derived BMMC in the co-culture, it is likely that cyclooxygenase pathway constitutes the additional pathway modulated by IL-1α. On the other hand, neutralization experiments using antibodies against IL-3, IL-4, IL-10, and NGF, indicated that these factors and co-factors for mast cell growth are unlikely to be involved.

It is consequently speculated that mast cell growth activity induced by IL-1α in fibroblasts is, at least in part, attributed to prostanoids, and that the signal conducted via SCF/c-*kit* pathway is important for this cytokine-induced mast cell growth activity, and/or SCF/c-*kit* interaction serves as adhesion molecules that keep mast cells in contact with or in close range of fibroblasts. Considering that the extracellular domain of c-*kit* expressed on *W/W$^v$*-derived BMMC is intact and can serve as adhesion molecule [27], SCF/c-*kit*-mediated signal seems to be essential for efficient fibroblast-dependent mast cell growth.

In time course experiments (Fig 1C), the effect of IL-1α was observed only in the earlier time period. Although it remains to be clarified, this limitation could be attributed

to the culture condition, in which contact-inhibited fibroblast monolayers were continuously stimulated with a mitogenic cytokine, which might affect the property of fibroblasts. Indeed, at the end of 14-d co-culture stimulated with higher concentration of IL-1α, fibroblasts showed a tendency to easily detach from culture dishes.

Hu et al. [28] reported that PGE is essential for IL-3-dependent mouse mast cell induction from spleen cells. The authors concluded that PGE acts on mast cell precursors, but not on committed immature mast cells. In human system, $PGE_2$ enhances mast cell differentiation from cord blood mononuclear cells by inhibiting the production of macrophage-derived GM-CSF, which induce the differentiation into granulocyte-macrophage lineage [29]. Our results indicate that $PGE_2$ also enhance the growth of differentiated mast cells in the co-culture system. It has been reported that keratinocyte-derived IL-1α induces increased $PGE_2$ production from dermal fibroblasts in human keratinocyte/fibroblast co-culture [30]. It is therefore possible that IL-1α-induced prostaglandin synthesis is involved in the mast cell growth mechanism in human skin.

In conclusion, our results indicate that inflammatory cytokines induce mast cell growth by a fibroblast-dependent mechanism. The data in a murine system might not exactly apply to the human system, however, our findings may lead to the elucidation of the pathogenesis of mast cell hyperplasia observed in inflammatory, not necessarily allergic, skin diseases.

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**Table 1**. Mitomycin C treatment of fibroblasts. Confluent monolayers of fibroblasts were incubated with 50 μg/ml of mitomycin C or medium at 37°C for 30 min. After thorough washing, BMMC were seeded on the monolayer and co-cultured in the presence of 10 ng/ml of IL-1α or control medium. Fibroblast and mast cell number was determined on day 7. Results were expressed as mean ± SEM of four experiments performed in quadruplicates.

| | Without mitomycin C treatment | | With mitomycin C treatment | |
|---|---|---|---|---|
| | Fibroblast number | Mast cell number | Fibroblast number | Mast cell number |
| Medium | 233439 ± 18078 | 11874 ± 2342 | 43279 ± 2339[a] | 9533 ± 2597 |
| IL-1α | 251270 ± 17520 | 25605 ± 2188[b] | 53035 ± 7552[a] | 25715 ± 1197[b] |

[a] $p<0.01$ versus cell numbers without mitomycin C treatment.

[b] $p<0.01$ versus medium control in each treatment group.

**Table 2**. Neutralization experiments with antibodies against IL-3, IL-4, IL-10, and NGF. BMMC were co-cultured with NIH/3T3 fibroblasts in the presence of 10 ng/ml of IL-1α, IL-4, IL-10, NGF, or 5 ng/ml of IL-3 for 6 d. Each antibody was added at a final concentration of 10 μg/ml. Growth of BMMC was determined by total cellular histamine (ng/well) and growth index was calculated as follows: (growth index) = (histamine content in each co-culture) / (histamine content in the control co-culture without cytokine). Data are expressed as mean ± SEM of growth index obtained in three experiments performed in duplicate. (ND not done)

| Cytokine in co-culture | Growth index | | | | |
|---|---|---|---|---|---|
| | Without antibody | With antibody | | | |
| | | anti-IL-3 | anti-IL-4 | anti-IL-10 | anti-NGF |
| IL-1α | 3.84 ± 0.87 | 2.82 ± 0.29 | 3.00 ± 0.71 | 3.42 ± 0.73 | 5.23 ± 1.14 |
| IL-3 | 3.24 ± 0.33 | 1.05 ± 0.05[a] | ND | ND | ND |
| IL-4 | 2.30 ± 0.28 | ND | 1.04 ± 0.07[a] | ND | ND |
| IL-10 | 3.37 ± 0.56 | ND | ND | 1.54 ± 0.09[a] | ND |
| NGF | 1.04 ± 0.06 | ND | ND | ND | 1.28 ± 0.07[a] |

[a] $p<0.05$ versus co-culture without antibody and stimulated with the same cytokine.

## Figure Legends

**Fig. 1** Mast cell growth induced by IL-1α in mast cell/fibroblast co-culture system. BMMC prepared from WBB6F$_1$-+/+ mice were seeded on the confluent monolayer of NIH/3T3 fibroblast and cultured in the presence of IL-1α or medium. A, B, Cells were harvested on day 7 and mast cell number (A) and total histamine content (B) was measured. C, Cells were cultured in the presence (closed circle) or absence (open square) of IL-1α (10 ng/ml), and harvested at each time point. Results are expressed as mean ± SEM of quadruplicates in a representative of four experiments for each study. *$p<0.05$ versus medium control at each time point.

**Fig. 2** Separation of mast cells from fibroblasts in the co-culture by using micropore membrane. BMMC were co-cultured with NIH/3T3 fibroblasts with or without tissue culture inserts attached with 0.2 μm pore membrane which separate mast cells from fibroblasts. Cells were cultured for 7 d in the presence of 10 ng/ml of IL-1α or medium alone. *$p<0.01$ versus medium control in each group.

**Fig. 3** Co-culture of BMMC derived from WBB6F$_1$-*W/W$^v$* mice on the NIH/3T3 fibroblast monolayer. BMMC prepared from WBB6F$_1$-*W/W$^v$* mice were co-cultured on NIH/3T3 fibroblasts in the presence of IL-1α (10 ng/ml), SCF (30 ng/ml), IL-3, 4 (5 ng/ml, 2 ng/ml, respectively) or medium. Mast cell number (A) and histamine content (B) were measured on day 7. Data are mean ± SEM of three experiments performed in quadruplicate. *$p<0.01$ versus medium control.

**Fig. 4** Co-culture of BMMC derived from WBB6F$_1$-+/+ mice with *Sl/Sl$^d$*-derived fibroblast monolayer. BMMC prepared from +/+-mice were co-cultured with *Sl/Sl$^d$*-derived fibroblasts in the presence of IL-1α (10 ng/ml), SCF (30 ng/ml), or IL-3, 4 (5 ng/ml, 2 ng/ml, respectively). Mast cell growth was determined by total cellular histamine on day 7. Data are mean ± SEM of three experiments performed in quadruplicate. *$p<0.05$, **$p<0.01$ versus medium control.

**Fig. 5** Inhibition of mast cell growth by indomethacin. BMMC derived from +/+- (A) and *W/W$^v$*- (B) mice were co-cultured on NIH/3T3 fibroblasts with 10 ng/ml of IL-1α or control medium either in the presence (closed column) or absence (open column) of 10 μM of indomethacin. Mast cell number (A) and total cellular histamine (B) were determined on day 6. Results are expressed as mean ± SEM of quadruplicates in a representative of three experiments. *$p<0.05$.

**Fig. 6** Mast cell growth induced by $PGE_2$ in the co-culture with NIH/3T3 fibroblasts. BMMC were co-cultured with NIH/3T3 fibroblasts in the presence of a series of $PGE_2$. Mast cell number (A) and total cellular histamine (B) were determined on day 6. Results are expressed as mean ± SEM of quadruplicates in a representative of three experiments. *$p<0.01$ versus medium control.

**Fig. 7** Co-culture of BMMC with skin-derived fibroblasts. BMMC obtained from WBB6F$_1$-+/+ mice were co-cultured on monolayers of skin-derived fibroblasts prepared from mice of the same strain in the presence of IL-1α or control medium for 7 d. Mast cell

growth was determined by total histamine content. Result of a representative of three experiments performed in duplicate is shown.

インターロイキン１α(IL-1a)は線維芽細胞依存的にマスト細胞の増殖を増強する。

マスト細胞はヒスタミンなどのケミカルメディエーターを遊離することによりアレルギー反応に重要な役割を演じるのみならず、種々のサイトカインを産生することにより、病態形成に積極的に関与するものと考えられ、アレルギー性疾患以外の種々の炎症性皮膚疾患においても増加することが知られている。皮膚マスト細胞は通常真皮上層に存在し、真皮の主要構成細胞である線維芽細胞がマスト細胞の増殖分化に重要な役割を果たしていることが in vitro では確認されているが、炎症性疾患におけるマスト細胞増殖機構については未だ解明されていない。本研究では、炎症性皮膚疾患におけるマスト細胞の増殖機構を解析するモデルとして、マスト細胞と線維芽細胞の共生培養系を用い炎症性サイトカイン IL-1a がマスト細胞の増殖に及ぼす効果を検討した。

マスト細胞としては、WBB6F1-+/+マウス骨髄を IL-3 存在下で培養して得られた骨髄由来培養マスト細胞(BMMC)を用い、線維芽細胞としては NIH/3T3 細胞を用いた。まず BMMC を NIH/3T3 シート上で培養する共生培養系にリコンビナントマウス IL-1a を添加したところ、IL-1a は 7 日間の培養において、10 pg/ml 以上の濃度で用量依存的にマスト細胞の増殖を増強し、また 10 ng/ml の IL-1a 存在下では、時間依存的な BMMC の増加が認められた。しかし BMMC を単独で培養した場合には、IL-1a を添加しても増殖反応はみられないことから IL-1a のマスト細胞に対する効果は線維芽細胞依存的であることが示された。さらに、IL-1a で 6 時間前刺激した NIH/3T3 上で BMMC を培養した場合には IL-1a を添加しなくても 3 日後の細胞数は対照に比し有意に高値であったのに対し、NIH/3T3 を IL-1a で刺激して得られた培養上清には、マスト細胞増殖活性はみられず、また共生培養系でマイクロポアーメンブレンによりマスト細胞と線維芽細胞の接触を妨げた場合には、IL-1a の効果は減弱した。以上の結果より、IL-1a は直接マスト細胞には作用せず、IL-1a により線維芽細胞からマスト細胞増殖因子が産生され、その因子によるマスト細胞増殖には両者の接着が重要であることが示された。

線維芽細胞は強力なマスト細胞増殖因子である Stem cell factor (SCF)を発現することが知られているため、この系における SCF の関与について検討した。SCF には遊離型、膜型が存在するが、上記の結果よりこの系においては膜型因子が重要であると推察されるが、フローサイトメトリーによる検討では、IL-1a による線維芽細胞上の SCF 発現増強は認められなかった。機能的検討として、SCF の受容体である c-kit に変異があり、SCF による増殖がみられない WBB6F1-W/Wv マウス由来の BMMC、あるいは膜型 SCF を欠損する Sl/Sld 線維芽細胞を用いて共生培養を行った場合には、IL-1a の効果は著しく減弱し、SCF の重要性を示すものと考えられた。しかしこの場合にも IL-1a による有意の効果がみられたことから、IL-1a によるマスト細胞増殖には、SCF 以外の因子が関わることが示された。

すでにマウスマスト細胞に対する増殖活性を持つことが知られている IL-3，あるいは SCF のコファクターとしてマスト細胞増殖を促進することが知られている IL-4，IL-10 の

関与についても検討した。リコンビナントマウス IL-3, IL-4, IL-10 を共生培養系に添加するとマスト細胞の増殖が認められたが、いずれに対する中和抗体も、IL-1a によるマスト細胞増殖を抑制しなかった。IL-3 を添加した共生培養系においてマスト細胞増殖活性が報告されている NGF については、我々の系（IL-3 非添加）では活性が認められず、また抗 NGF 抗体による IL-1 の効果の抑制も認められなかった。従って IL-1a によるマスト細胞増殖には既知の因子以外が関与することが推定された。共生培養系にシクロオキシゲナーゼインヒビターインドメサシンを加えると IL-1a の効果が抑制されたことから、シクロオキシゲナーゼ、およびその産物であるプロスタノイドが役割を演じていることが示唆され、実際 PGE1 が共生培養系において用量依存的にマスト細胞増殖を示すことが確認された。

以上の結果から、共生培養系における IL-1a によるマスト細胞増殖には、SCF-c-kit を介する経路が重要であり、さらに IL-1a により線維芽細胞に誘導される因子が存在すること、その第２の経路にプロスタノイドが関与していることが示された。

NIH/3T3 線維芽細胞はマウス胎児由来の線維芽細胞であるが、マスト細胞用の骨髄を採取したマウスと同系の WBB6F1-+/+マウスの皮膚より採取した線維芽細胞との共生培養の系においても同様の効果が認められた。

従って、炎症性サイトカイン IL-1a は皮膚におけるマスト細胞の増殖を惹起しうると考えられ、炎症性皮膚疾患、必ずしもアレルギー性に限らず、の際に認められるマスト細胞の増加の機構の一端を解明する結果と考えられる。

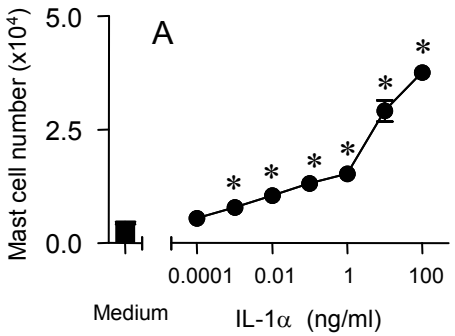


Fig 1A
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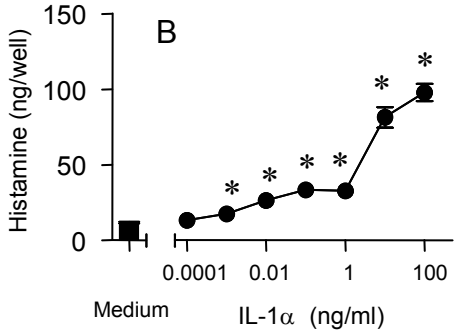


Fig 1B
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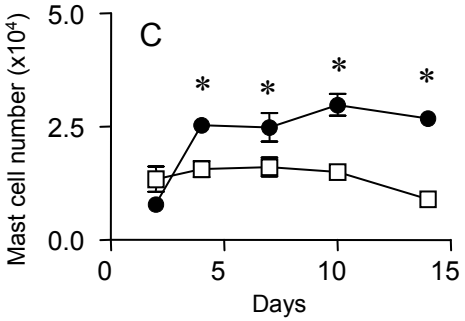


Fig 1C
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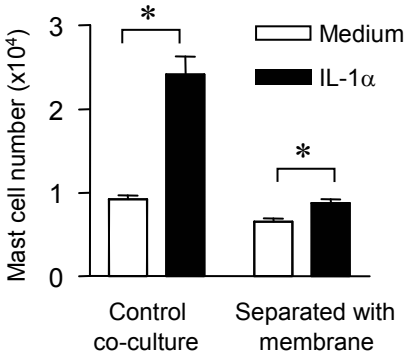


Fig 2
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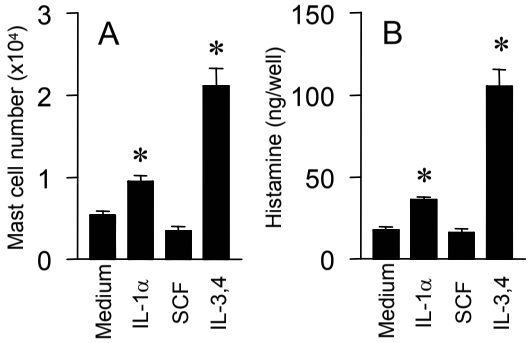


Fig 3
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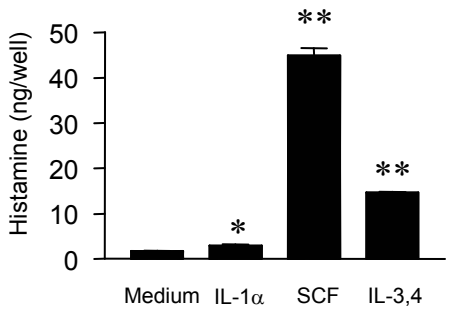


Fig 4
Kameyoshi et al.

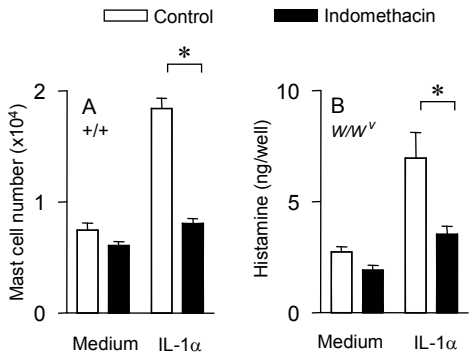


Fig 5
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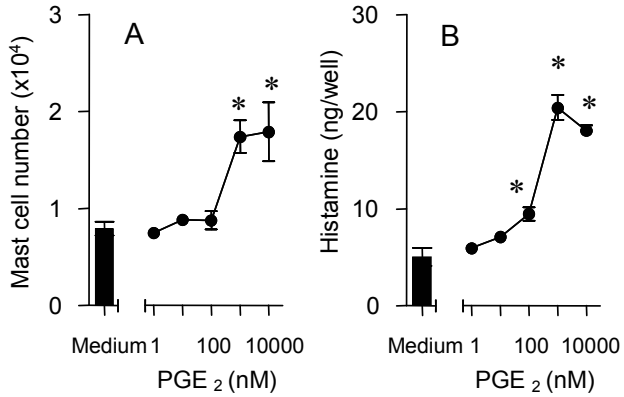


Fig 6
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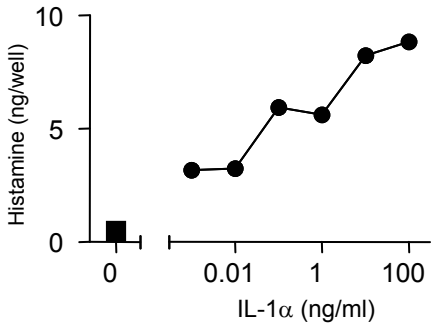


Fig 7
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